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Manuscript title: High tidal volume ventilation is not deleterious in infant rats exposed to severe hemorrhage

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Abstract and key words

Background: Both high tidal volume (V_T) ventilation and hemorrhage induce acute lung injury in adult rodents. It is not known whether injurious ventilation augments lung injury in infant rats exposed to severe hemorrhage.

Methods: Two week old rats were allocated to ventilation with V_T 7 mL/kg and PEEP 5 cmH_2O (low V_T) or V_T 21 mL/kg and PEEP 1 (high V_T) for 4 h. Additional rats were subjected to volume-controlled hemorrhage and delayed saline resuscitation, followed by low V_T or high V_T ventilation for 4 h. Non-ventilated control groups were also included. Airway resistance and the coefficient of tissue elastance (H) were derived from respiratory input impedance measurements using the low-frequency forced oscillation technique. Pressure-volume curves were obtained at baseline and at the end of the study. Interleukin-6 (IL-6), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor alpha (TNF-α) were determined in bronchoalveolar lavage fluid (BALF) and serum.

Results: In both healthy and hemorrhage-exposed animals high V_T resulted in reduced H (better lung compliance), and increased transcutaneous oxygen saturation. IL-6 in BALF was greater in ventilated animals when compared to non-ventilated controls, but not different between ventilated groups. No significant differences were found for all other inflammatory mediators, total protein concentration in BALF, and histology.

Conclusion: High V_T ventilation with low PEEP improves respiratory system mechanics without causing additional damage to healthy and hemorrhage-exposed infant rats after 4

h of ventilation. This study highlights the tolerance to high V_T ventilation in infant rats and underscores the need for age-specific animal models.

Keywords: hemorrhage, high tidal volume, ventilator-associated lung injury, respiratory system mechanics, infant rat, macrophages, pediatric mechanical ventilatory support, pediatric VALI

1. Introduction

Recommendations for mechanical ventilation strategies in infants and children are based on evidence from human and animal adult data, since both solid data from age-specific animal models and large randomized controlled studies from pediatric populations are lacking.[1-3] Hence, current adopted lung protective ventilation strategies in children also recommend the use of low tidal volume (V_T), limitation of peak inspiratory pressure, and adequate positive end-expiratory pressure (PEEP) to avoid volutrauma and atelectrauma and minimize biotrauma.[4-6] However, given the developmental and physiological differences between children and adults, it is not clear whether adult data can be extrapolated to children with respiratory failure. In fact, evidence from in vivo rat studies suggests that healthy infant rats better tolerate short-term high V_T ventilation with zero PEEP than adult rats.[7,8]

Infant rats provide a useful model to investigate pediatric lung injury because of characteristic developmental changes that are similar to those in human lungs.[9] In terms of lung development and alveolarization 2 week old rats correspond to 1-2 year old human infants.[10,11] Ventilator-associated lung injury (VALI) has been studied in infant rats with healthy lungs[7,8,12] or after lipopolysaccharide exposure[13] using V_T ventilation ranging from 25 to 40 mL/kg and 0 cmH_2O PEEP. However, it is not clear how infant rats respond to high but less extreme lung stretch over a longer period of time when compared to low V_T ventilation with high PEEP. Also, it has not been investigated in infant rats whether mechanical ventilation aggravates inflammatory response in pre-existing systemic inflammation induced by severe hemorrhage. Investigating this

interaction is clinically particularly important for surgical patients experiencing acute blood loss after trauma or operations.

In this study, we sought to address how high V_T ventilation with low PEEP affects lung mechanics in infant rats when compared to a theoretically protective strategy with low V_T and high PEEP. We hypothesized that high V_T ventilation with low PEEP, resulting in overdistension and shear stress produces lung injury and that prior hemorrhage-exposure potentiates inflammatory response.

2. Materials and Methods

Experimental protocols were approved by the local Animal Experimentation Ethics Committee and were performed in accordance with Australian guidelines. Rat pups were kept under 12 h light and dark cycle and were housed with their parents and littermates.

2.1 Preliminary studies

Estimates of blood volume in adult rats vary with strain, gender, and technique to between 5.2-7.8 mL/100 g body weight.[14-17] We assumed, taking into account that younger animals have increased circulating blood volumes, that blood volume approximated 7% of body weight. We preferred a volume-controlled hemorrhage model over a mean arterial pressure-guided model, since the latter is greatly affected by response to anesthetics.

Removing up to 30% of blood volume followed by fluid resuscitation with saline did not cause any signs of distress or affect behavior and weight gain after 24 and 48 h when compared with age-matched controls (data not shown). Controlled hemorrhage beyond 30% of estimated blood volume was difficult to achieve by tail tipping in adequately anesthetized infant rats due to reduced peripheral blood flow. However, the withdrawn ~0.55 mL blood in ~26 g infant rats was relatively close to the amount of blood we could take by direct cardiac puncture (i.e. ~0.65 mL) in control groups. Lastly, we tested whether delayed fluid resuscitation 60 min after removal of 30% blood volume had an impact on distress or survival in the first 48 h. These animals behaved normally with similar weight gain to control rats. Based on these preliminary studies we chose a hemorrhage model with 30% withdrawal of estimated blood volume and delayed fluid

resuscitation, since our goal was both to use a clinically relevant hemorrhage model and render the animal capable of withstanding further mechanical ventilation.

2.2 Animal preparation before mechanical ventilation

After inhalational anesthesia with methoxyflurane 2 week old PVG rats (26.3 ± 2.2 g) underwent volume-controlled hemorrhage by tail tipping over 5 min. Then, infant rats recovered in a warm environment and had to be fully responsive before being returned to the parental cage. Sixty min later, 1.5x the amount of blood withdrawn was injected i.p. as saline solution. Non-ventilated control groups underwent the same anesthetic procedures without tail tipping, blood withdrawal, and fluid resuscitation.

Thirty min later, infant rats were anesthetized with an i.p. injection of a solution containing ketamine (80 µg/g), xylazine (13 µg/g), and saline (further anesthetic was given as required). A tracheostomy was performed and a 10 mm polyethylene cannula (ID: 0.86 mm) inserted. The rat was then placed in supine position on a heating mat and connected to a computer-controlled ventilator (*flexiVent*®, Scireq, Montreal, Canada) using following settings: inspired oxygen fraction (FiO_2) 0.4, respiratory rate (RR) 90/min, V_T of ~ 7 mL/kg, and PEEP 3 cmH_2O. Heart rate and transcutaneous oxygen saturation ($S_{tc}O_2$) were monitored via oximeter (MouseOx™, STARR Life Sciences Corporation™, Oakmont PA, USA) by placing a non-invasive sensor on the tail.

2.3 Measurement of respiratory system mechanics and allocation to study groups

Airway opening pressure was recorded by the *flexiVent*® system via a pressure transducer located at the Y-tubing connecting the endotracheal tube and the inspiratory and

expiratory ports. Lung volume history was standardized by application of 2 lung volume recruitment maneuvers with 40 mL/kg within 2 min. Then, following a pressure-volume (PV) curve from 3 to 20 cmH_2O, baseline measurement of respiratory system input impedance (Z_{rs}) was performed using the low-frequency forced oscillation technique provided by *flexiVent*® system. Z_{rs} was obtained with a 4 s broadband signal between 1.0 and 20.5 Hz during a pause from mechanical ventilation. The "constant-phase" model was fitted to the resulting Z_{rs},[18] allowing the estimation of airway resistance (R_{aw}) and inertance, and the coefficients of tissue damping and elastance (H). For the purpose of this study only R_{aw} and H are reported. Subsequently, rats were randomly allocated to one of the following ventilation strategies:

Study groups 1 and 2 (low V_T high PEEP, n=10 per group): V_T ~7 mL/kg, PEEP 5 cmH_2O, RR 90/', FiO_2 0.4 in control animals and after hemorrhage, respectively; study groups 3 and 4 (high V_T low PEEP, n=10 per group): V_T ~21 mL/kg, PEEP 1 cmH_2O, RR 30/', FiO_2 0.4 in control animals and after hemorrhage, respectively. We increased FiO_2 if $S_{tc}O_2$ fell below 90% for more then 60 s and remained low even after repositioning of the sensor. Animals were ventilated for 240 min with Z_{rs} measurements at baseline and every 60 min. In order to avoid dehydration 0.3 mL of saline solution was given i.p. after the 60 and 180 min measurements. Body temperature was maintained at 36.5-37.5° C with a heating pad. Study groups 5 and 6 (n=5 per group) included non-ventilated controls and non-ventilated hemorrhage exposed animals, respectively.

After the last Z_{rs} measurement, ventilation strategy was put back to V_T ~7 mL/kg, PEEP 3 cmH_2O, and RR 90/min. One min later, another PV curve was performed before the animal was disconnected from the ventilator.

2.4 Sampling and processing of bronchoalveolar lavage fluid (BALF) and serum

For lung lavage at the end of the protocol 0.8 mL of 0.9% saline solution were instilled in and out of the lung 3x and ice cooled until centrifugation at 2000 r/min for 4 min. Supernatant was collected and frozen for future analysis of macrophage inflammatory protein-2 (MIP-2), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and total protein. The cell pellet was resuspended in phosphate buffered saline and an aliquot was stained with Trypan blue to obtain a total cell count. A cytospin of the remaining pellet was stained with Leishman's for differential counting using light microscopy. Blood samples obtained via direct cardiac puncture were allowed to clot before centrifugation at 2000 r/min for 10 min. Serum was frozen for later analysis of IL-6, MIP-2, and TNF-α. Cytokine concentrations were measured by using specific ELISA, following the manufacturer's instructions (BD Biosciences, San Diego, California). Total protein was analyzed using a colorimetric protein assay (Bio-Rad Inc, Regents Park, NSW, Australia).

2.5 Morphologic analysis of lung tissues

Lungs were fixed by 10% phosphate buffered formalin (PBF) instillation via the endotracheal tube at a pressure of 10 cmH_2O. Two h later lungs and heart were removed from the thoracic cavity and stored in a PBF filled container. At the time of processing the heart was dissected free and the remaining tissues were processed whole in paraffin, and embedded with the caudoventral aspect down. Five μm thick sections were cut from the caudoventral aspect to include as many lung lobes as possible, and stained routinely

with haematoxylin and eosin. A specialist veterinary pathologist (PN), blinded to the treatments, assessed slides (n=5 per group) by light microscopy for interstitial inflammation, number of inflamed foci, neutrophil inflammation, macrophages, and edema. Neutrophils and macrophages were counted in ten fields, distributed across multiple lung lobes, under the x40 objective.

2.6 Statistical analysis

For group comparison of BALF and serum outcome parameters, $S_{tc}O_2$, heart rate, peak airway opening pressure (P_{ao}), R_{aw} and H, and PV curves two-way ANOVA (strategy and treatment) with Holm-Sidak post-hoc tests were used. One-way repeated measures ANOVA with Holm-Sidak post-hoc tests were used to compare P_{ao} and Z_{rs} changes over time within study groups. A paired t-test was used to compare heart rate, $S_{tc}O_2$, and PV curves at selected time points. Data were transformed where appropriate to ensure the assumptions of normality and equal variance were satisfied. Where this was not possible equivalent non-parametric comparisons were used. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Airway resistance (R_{aw}) and tissue elastance (H)

No significant changes were seen for R_{aw} between the groups at baseline or during the study period (Fig. 1A). H is illustrated in Fig. 1B. Hemorrhage-exposed animals had slightly elevated values at baseline ($p=0.006$). Over time, H linearly rose in animals ventilated with low V_T, whereas high V_T ventilation resulted in an approximately exponential increase in H that approached a plateau after the first hour of ventilation. Though both ventilation strategies resulted in significant rises of H when compared with baseline, low V_T ventilation produced significantly greater H values at the end of the study. The changes in H over time were related to the ventilation pattern and not influenced by blood loss.

3.2 Peak airway opening pressure (P_{ao})

Changes in peak P_{ao} are displayed in Fig. 2. At baseline, peak P_{ao} was not different between infant rats ($p>0.41$ in all cases). When compared to the substantial impact of high V_T ventilation on peak P_{ao} at the time point 60 min, PEEP elevation from 3 to 5 cmH_2O in both low V_T groups produced a significant but relatively moderate rise in peak P_{ao}. After that, low V_T ventilation resulted in a significant and continuous increase of peak P_{ao}, whereas high V_T ventilation produced stable peak P_{ao} values ($p>0.18$ in high V_T groups).

3.3 Pressure-volume curves

PV curves were similar before allocation to study groups (Fig. 3A) ($p>0.34$ in all cases). Four h later, high V_T ventilation resulted in significantly better compliance of the inflation and deflation limb when compared with low V_T ventilation (Fig. 3B). Comparison within single study groups revealed that lung volumes at an inflation pressure of 20 cmH_2O fell by ~35% in both low V_T groups, whereas high V_T ventilation only produced a decrease by 11% and 2.5% in controls and hemorrhage-exposed animals, respectively.

3.4 Total and differential cell count, and total protein concentration in BALF

As shown in Fig. 4, the number of total cells and macrophages were significantly higher in non-ventilated control groups when compared with all other study groups. No significant differences were found between all other groups ($p>0.052$ in all cases). The number of recovered neutrophils from BALF was significantly greater in ventilated animals. However, no differences were found between ventilated controls and hemorrhage-exposed groups ($p>0.65$ in all cases). Concentrations of total protein in BALF did not differ between groups ($p=0.13$).

3.5 Cytokine concentrations in BALF and serum

Concentrations of MIP-2 in BALF and IL-6 in serum did not significantly differ between study groups ($p=0.45$ and $p=0.13$, respectively) (Fig. 5). Though IL-6 concentrations in BALF were significantly increased in ventilated groups when compared to non-ventilated groups, there was no significant difference between ventilated groups ($p>0.50$ in all

cases). TNF-α concentrations in BALF and MIP-2 and TNF-α concentrations in serum were below detection levels of the ELISA kits (<60, 34, and 140 pg/mL, respectively).

3.6 Transcutaneous oxygen saturation and heart rate

At baseline we found no significant differences for $S_{tc}O_2$ and heart rates between study groups ($p>0.15$ in all cases) (Table 1). Only $S_{tc}O_2$ of high V_T ventilated control animals rose significantly over time. For the last 30 to 60 min 3 out of 10 and 1 out of 10 hemorrhage-exposed animals ventilated with low V_T and high V_T, respectively, required increases in FiO_2 up to 0.8. To account for this intervention we also calculated the ratio $S_{tc}O_2/FiO_2$. Over time, heart rates significantly decreased in animals ventilated with low V_T, whereas ventilation with high V_T resulted in stable heart rate ($p>0.10$).

3.7 Morphologic analysis of lung tissues

Histologic assessment of lung tissue samples revealed normal lung structures in all study groups with minimum scores in both non-ventilated and ventilated animals (data not presented).

4. Discussion

The main findings of this study were as follows. First, high V_T ventilation in combination with low PEEP resulted in an overall improvement of respiratory system mechanics when compared to low V_T high PEEP ventilation. Second, contrary to our hypothesis, high V_T ventilation did not enhance inflammatory response after 4 h of mechanical ventilation. Third, induction of severe hemorrhage prior to mechanical ventilation neither predisposed rats to VALI nor resulted in a greater inflammatory mediator release.

Mechanical ventilation strategies aim at preventing and minimizing volutrauma, atelectrauma, and biotrauma. In adult rats with primarily healthy lungs injurious effects of high V_T ventilation[19] and very low PEEP[20] resulting in pulmonary edema, alveolar instability, and increased cytokine release[21] have been demonstrated. Comparison of adult and infant rats exposed to a harmful ventilation mode with very high V_T ventilation and zero end-expiratory pressure (ZEEP) revealed tolerance of infant rats towards high V_T ventilation after 180 min.[7,8] Whether ventilation was based on V_T or peak inspiratory pressure younger animals showed better lung compliance, less cytokine production, and lower histopathologic scores.

In the first part of the present study, we compared a low V_T-high PEEP to a high V_T-low PEEP ventilation strategy. The former strategy was designed to minimize repeated opening and closing of peripheral lung units, while the latter one complied with theoretical requirements for development of volutrauma and atelectrauma.[6] Mechanical ventilation with low V_T resulted in significantly higher H values than high V_T ventilation. The linear increase in H is compatible with a progressive loss of lung volume as a result of progressive airway closure[22-24] that was not prevented by a PEEP of 5 cmH_2O. In rats

ventilated with low V_T neither V_T nor peak P_{ao} was probably high enough to reopen atelectatic lung units and reverse the increase in H over time. In contrast, high V_T ventilation initially led to a steep rise in H, followed by stable H values during the second half of the protocol. The initial and almost linear increase in H can be explained by rapid loss of lung volume due to low PEEP levels.[24] Further increase in H was most likely prevented by alveolar recruitment of peripheral lung units via application of high V_T ventilation resulting in peak P_{ao} of ~19.5 cmH_2O (versus ~12.7 cmH_2O in the low V_T group). The PV curves obtained at the end of the study corroborate the assumption that high V_T ventilation provided greater lung volumes when compared to low V_T groups. Moreover, decrease in heart rate and lower $S_{tc}O_2$ observed after low V_T ventilation probably reflect considerable ventilation-perfusion mismatch resulting from progressive airway closure.

The role of surfactant in lung compliance has been invoked in both adult[20] and infant rat studies.[12] Verbrugge et al[25] found that significant changes in surfactant composition and function occurred after application of high V_T on top of ZEEP, and setting an adequate PEEP prevented surfactant dysfunction. Martinez et al[12] using a neonatal rat model pointed at stretch-induced surfactant release to explain improved lung compliance after ventilation with extreme V_T of 40 mL/kg and ZEEP. However, the increase of the active subfraction of surfactant peaked at 60 min and was short-lived. The role of surfactant has not been investigated in our study. We speculate that infant (non-neonatal, non-adult) animals react differently to stretch-induced stimuli and that surfactant production and uptake last longer.

We designed the second part of the present study to assess the interaction between mechanical ventilation and antecedent hemorrhage. We chose to delay fluid resuscitation because of two reasons. First, immediate fluid resuscitation is not always possible in clinical situations. Second, there is evidence that delayed resuscitation produces a more prominent inflammatory reaction with elevated cytokine concentrations in serum (i.e. IL-6 and TNF-α) and more histologic lung injury.[26]

Although resuscitation from severe hemorrhage can trigger acute lung injury, the exact mechanism is not clear. Tissue ischemia and reperfusion leading to production of reactive oxygen species and neutrophil activation,[27,28] impaired alveolar perfusion causing local damage and ventilation-perfusion mismatch,[29] and increased inducible nitric oxide synthase[30] have been proposed as important mechanisms. The two-hit hypothesis, whereby severe hemorrhage and resuscitation act as a first and mechanical ventilation as a second hit was supported in adult rat studies.[31-33] In these animals high V_T ventilation and ZEEP, as opposed to low V_T with PEEP, augmented cytokine response (TNF-α, IL-1β, IL-6, and MIP-2) and increased lung elastance, and histologic lung injury scores.

In the present study, priming with severe hemorrhage followed by resuscitation did not render the lungs more susceptible to mechanical ventilation. Irrespective of applied ventilation strategy we measured significantly increased neutrophil numbers and IL-6 concentrations in BALF of all ventilated animals. In contrast to results reported by Boudma et al,[32] both low V_T and high V_T ventilation alone increased BALF IL-6 when compared to non-ventilated controls. Differences when compared to VALI adult rat studies were also found after histological assessment of lung tissue samples. While

neutrophil infiltration and edema were reported after high lung stretch and/or severe hemorrhage in adult rats,[26,33,34] this was clearly not the case in the present study. Furthermore, MIP-2, a rodent homologue to the chemokine IL-8 and potent neutrophil chemoattractant,[35] was not affected by hemorrhage and resuscitation or mechanical ventilation. A similar cytokine profile with elevated IL-6 and unchanged MIP-2 concentrations in BALF was reported in a clinical study conducted in infants before and after short-term mechanical ventilation.[36] The role of IL-6 and MIP-2 as mediators in the pathogenesis of acute lung injury in infants remains unclear and warrants further investigation.

Interestingly, the number of total cells and macrophages recovered from BALF was significantly greater in non-ventilated controls when compared to all other groups. This finding deserves closer consideration. First, acute blood loss might result in a decreased number of BALF macrophages via migration to the primary site of injury or inadequate supply of precursor monocytes from the peripheral circulation.[37] Second, alveolar macrophages detect and respond to mechanical stress by release of cytokines such as TNF-α, IL-8, and IL-6.[38] Hence, mechanical forces applied during artificial ventilation might be converted into early proinflammatory signals resulting in activation and adhesion of macrophages to the alveolar epithelium.[39,40] Decreased macrophage number recovered from lavage fluid after injurious ventilation has been reported previously.[39-42] We did not find significant differences in the number of recovered BALF cells and cytokine response between low and high V_T groups, but low V_T ventilation clearly resulted in impaired lung function. Based on these findings, it seems reasonable to suggest that low V_T ventilation of infant rats also causes significant mechanical stress

presumably via atelectasis-related shear stress. Third, apoptosis and necrosis during the process of lung injury are also possible causes for the decreased macrophage number derived from BALF in hemorrhage exposed and ventilated animals.[39-41] Since we did not directly assess migration, adhesion, or apoptosis of alveolar macrophages, based on our findings, we can only speculate that mechanical ventilation induced macrophage activation and increased IL-6 production with subsequent neutrophil recruitment.

The following study limitations merit further comments. First, plateau pressure as the best indicator of overdistension has been proposed rather than V_T, since the majority of VALI studies in adult animals only demonstrated lung injury after high or excessive V_T ventilation when plateau pressure was well above 25 cmH_2O.[43] However, Copland et al[7] comparing the effects of high V_T with 25 mL/kg in adult and infants rats clearly showed that lung damage occurred after 90 min in adult animals ventilated at peak inspiratory pressures below 20 cmH_2O. Second, selection of a volume-controlled hemorrhage model and the amount of fluid replacement may partly explain the differences between our study results and those obtained by others using a mean arterial pressure-guided hemorrhage model.[31-33]

In conclusion, we have demonstrated in intact infant rats that high V_T ventilation with low PEEP improves respiratory system mechanics without causing additional damage. In addition, we have shown that antecedent severe hemorrhage followed by delayed fluid resuscitation does not augment susceptibility to mechanical ventilation associated injuries. This study highlights the tolerance of infant rats to a ventilation strategy (high V_T and low PEEP) that is known to induce lung injury in adult rats. While

it is too early to extrapolate these findings to clinical practice, our results underscore the need for age-specific animal models in the studies of pediatric VALI.

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7. Figure legends

Figure 1

Panels A and B display airway resistance (R_{aw}) and the coefficient of tissue elastance (H), respectively, as a function of time on the ventilator. At each time point 4 respiratory system input impedance (Z_{rs}) spectra were collected within 120 s and the corresponding values for R_{aw} and H were averaged. Data are expressed as group means ± standard error of the mean (n=10 per group). At the end of the protocol significant differences * between study groups were found for H. Differences were related to strategy, i.e. high versus low tidal volume ventilation ($p<0.001$), and not to treatment ($p=0.42$).

Figure 2

Peak airway opening pressure (P_{ao}) as a function of time. Data are expressed as group means ± standard deviation (n=10 per group).

Figure 3

Panels A and B show changes in volume plotted against airway opening pressure at the beginning and end of the protocol, respectively. Data are expressed as group means ± standard deviation (n=10 per group). At the end of the study we found a significant difference * between high and low V_T ventilation ($p<0.001$), with no significant difference related to antecedent hemorrhage ($p=0.43$).

Figure 4

Figure 4 illustrates total and differential cell counts in BALF. Data are presented as median with interquartile ranges (n=10 per group).

Figure 5

Figure 5 shows macrophage inflammatory protein-2 (MIP-2) and interleukin-6 (IL-6) concentrations in bronchoalveolar lavage fluid (BALF), and IL-6 concentrations in serum. Data are presented as median with interquartile ranges (n=10 per group). * indicates statistically significant differences between ventilated and non-ventilated groups; no statistically significant differences were found between ventilated study groups ($p>0.50$ in all cases).